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<p>The invention provides methods of treating a variety of diseases and conditions by administering an effective amount of an integrin modulating agent, as further defined and specified herein, to an animal suffering from one of the diseases or conditions. The preferred integrin modulating agents are ajoene and disintegrins, variants and analogs of disintegrins. In particular, the invention provides: (1) a method of treating inflammation; (2) methods of treating and preventing viral infections; (3) a method of treating shock; (4) a method of treating arthritis; (5) a method of contraception; (6) a method of suppressing immune responses; (7) a method of treating an autoimmune disease; (8) a method of inhibiting undesirable integrin-mediated cell-cell fusion; (9) a method of inhibiting the formation of lesions; (10) a method of treating psoriasis; (11) a method of treating atherosclerosis; (12) a method of treating diseases or conditions involving a plurality of integrin-dependent etiopathogenetic mechanisms; and (13) a method of inhibiting the transfer of genetic material.</p>			

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METHODS OF USING INTEGRIN MODULATORS

BACKGROUND OF THE INVENTION

5 Integrins are heterodimeric transmembrane glycoproteins which act, inter alia, as cell receptors for surface molecules of other cells and for extracellular matrix (ECM) proteins, herein termed collectively "integrin ligands." Both soluble and
10 immobilized integrin ligands are known to be ordinarily bound by integrins. Integrins are found on most types of cells. Ligand binding by integrins may result in a series of additional cellular events. When these additional events occur, they involve one
15 or more cellular functions. These cellular events and functions, some of which are discussed below for illustrative purposes, are integrin-mediated. For a general review of integrins, see, Guidebook to the Extracellular Matrix and Adhesion Proteins (Kreis, et
20 al., Eds.), 1993, and Pigot, et al., The Adhesion Molecule Facts Book, Academic Press, 1993.

 One such integrin-mediated cellular function is signaling. For instance, certain integrins are known to transfer information from the inside to the
25 outside of the cell (inside-out signaling), and/or from the outside to the inside of the cell (outside-in signaling), although other types of signaling may also occur. An example of inside-out signaling is the process whereby an integrin acquires affinity for
30 ligands in response to cell activation-associated

intracellular events (integrin upregulation). Binding of integrin ligands to certain integrins (e.g., in the case of integrin-mediated cell adhesion) may initiate signal transduction events, in a manner similar to that described for other cell surface receptors. The signals thus elicited are termed outside-in signals and regulate various cell responses, such as gene expression, cell differentiation, and cell proliferation.

Signaling may result in integrin clustering, i.e., the association of integrins with each other (and other molecules) by lateral interactions. The formation of such clusters may influence various integrin functions in multiple ways, including, for example, by secondary signaling.

The integrin-mediated function of adhesion is important for a variety of physiological and pathological responses. The extent of adhesion is controlled by integrin signaling. For example, as a result of initial integrin-dependent adhesion to a substratum, certain cells change their shape and start spreading on its surface, using integrins for establishing new contacts with the underlying proteins (e.g., ECM components). In motile cells, the whole array of integrin-mediated events involving adhesion -- initial contact, cell shape change, cell spreading, and cell locomotion -- is sometimes termed "the adhesion cascade" (Sharar, S.R., et al., The Adhesion Cascade and Anti-Adhesion Therapy: An Overview, 16 Springer Semin. Immunopathol. 359, 1995). Examples of adhesion cascades include tumor cell metastasis, cell migration processes associated with wound healing, and lymphocyte homing, although similar cascade mechanisms are operative in the absence of locomotion (e.g., platelet adhesion and aggregation). Extravasation of neutrophils is described below in greater detail, as a

paradigmatic integrin-driven adhesion cascade (Hub, E., et al., Mechanism of Chemokine-Induced Leukocyte Adhesion and Emigration, Chemoattractant Ligands and Their Receptors (Horuk, R., Ed.), Boca Raton, CRC Press, 1996, 301).

The onset of extravasation is signaled by the appearance in the circulation of chemotactic factors, or chemoattractants (i.e., specific substances that initiate cell migration along their concentration gradients). Chemoattractants (e.g., chemokines, bacterial peptides, and products of complement activation) activate neutrophils and upregulate their integrin receptors (neutrophil integrins include, e.g., LFA-1 [CD11a/CD18], CR3 [also known as Mac-1, CD11b/CD18], and gp150,95 [CD11c/CD18].) As a result, the activated neutrophils adhere to endothelial cells, change shape, and spread on the endothelial surface. Thereafter, the motile apparatus of the neutrophils is stimulated by the chemoattractants, and they start migrating, first across the endothelial layer and further, through the perivascular ECM, towards the source of the chemotactic stimulus, e.g., pathogenic bacteria invading a certain bodily tissue. During the whole process, from the initial firm contact with the endothelium to the cessation of locomotion at the destination site, various integrins serve to attach the neutrophil to the substrata it encounters, enabling its recruitment to the locus of infection.

Another integrin-mediated function is cell-to-cell fusion. Under physiological conditions, fusion is a developmentally regulated stage in the differentiation of certain multinucleate cells (e.g., osteoclasts, myocytes, and syncytiotrophoblasts) and a prerequisite to fertilization (in the case of sperm-egg fusion). Fusion is effected by specialized

cellular systems involving integrins (see, e.g., refs. cited in Huovila, A.-P.J., et al., ADAMs and Cell Fusion, 8 Current Opin. Cell. Biol. 692, 1996 and Ohgimoto, S., et al., Molecular Characterization of Fusion Regulatory Protein-1 [FRP-1] that Induces Multinucleate Giant Cell Formation of Monocytes and HIV gp160-Mediated Cell Fusion: FRP-1 and 4F2/CD98 Are Identical Molecules, 155 J. Immunol. 3585, 1995).

The ability to undergo recirculation from intracellular compartments to the cell surface and vice versa is a common property of diverse cellular receptors, including integrins (see, e.g., Handagama, P., et al., Kistrin, an Integrin Antagonist, Blocks Endocytosis of Fibrinogen into Guinea-Pig Megakaryocyte and Platelet α -Granules, 91 J. Clin. Invest. 193, 1993). This capability of integrins serves to mediate other cellular functions by transporting into the cell extracellular material (e.g., soluble proteins, particulate matter, and other cells). Integrin-mediated internalization is used by certain microorganisms to invade their targets. For example, CR3 mediates entry of iC3b-opsonized HIV-1 and HIV-2 into CD4-negative lymphocytic and monocytic cells (Boyer, V., et al., Complement Mediates Human Immunodeficiency Virus Type 1 Infection of a Human T cell Line in a CD4- and Antibody-Independent Fashion, 173 J. Exp. Med. 1151, 1991).

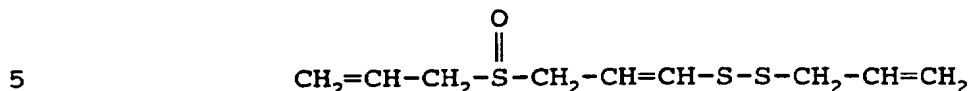
The above-delineated functions of integrins are illustrative only, as other characterizations of integrin functions can also be made. Moreover, the integrin-mediated functions as delineated herein are overlapping and interrelated. In the case of neutrophil extravasation, for example, the initial chemotactic signal activating the cells results in integrin upregulation (inside out signaling) and adhesion to the endothelial surface. This adhesion

event, in turn, elicits an outside-in signal, enabling the neutrophil to change its shape, which is a prerequisite to spreading and migration. Likewise, when the neutrophil that has arrived to the source of chemoattractants adheres to the bacteria, an outside-in signal transduced via the involved integrins initiates their internalization, together with the attached bacteria (phagocytosis).

Furthermore, regarding outside-in integrin signaling, certain cellular processes are coregulated by several distinct signaling systems acting in a concert. In the case of neutrophils extravasating to the tissues to phagocytose bacteria, the cells receive signals via distinct integrins (first from those that attach it to the substratum and subsequently from those recognizing the bacteria) and the receptors of the chemoattractant (along the concentration gradient of which the movement occurs). This interplay of signals regulates the antibacterial machinery of the neutrophils in such a way that only upon contact with the bacteria, which is established via a particular type of integrin, are the constituents of the intracellular granules released and reactive oxygen species formed. As a result, the formation and release of microbicidal substances take place preferentially at sites of contact with bacteria, enabling their effective killing and preventing the destruction of host tissue (Wright, S.D., Receptors for Complement and the Biology of Phagocytosis [Chapter 25], Inflammation: Basic Principles and Clinical Correlates [Gallin, J.I., et al., Eds.], 2nd Ed., New York, Raven Press, 477, 1992).

The present invention involves the regulation of a broad range of cellular activities by modulating certain integrin functions. One prototype integrin modulator of the present invention is Ajoene.

Ajoene is 4,5,9-trithiadodeca-1,6,11-triene-9-oxide, having a structural formula as follows:



Ajoene, and a precursor thereof, can be isolated from extracts of garlic (*Allium sativum*). As the garlic is crushed, alliin in the garlic comes into contact with allinase in the cell wall to form
10 allixin. Then, in the presence of a polar molecule, such as a lower alcohol or even water, allixin forms Ajoene.

Ajoene has been previously shown to inhibit platelet aggregation by inactivating allosterically the platelet integrin, GP IIb/IIIa (Apitz-Castro, R., et al., 141 Biophys. Res. Commun. 145, 1986). This inhibition of integrins by Ajoene is reversible.

SUMMARY OF THE INVENTION

20 The invention provides methods of treating a
variety of diseases and conditions by administering an
effective amount of an integrin modulating agent, as
further defined and specified below, to an animal
suffering from one of the diseases or conditions. The
25 preferred integrin modulating agents are Ajoene and
disintegrins, and variants and analogs of
disintegrins.

In particular, the invention provides: (1) a method of treating inflammation; (2) methods of treating and preventing viral infections; (3) a method of treating shock; (4) a method of treating arthritis; (5) a method of contraception; (6) a method of suppressing immune responses; (7) a method of treating an autoimmune disease; (8) a method of inhibiting undesirable integrin-mediated cell-cell fusion; (9) a method of inhibiting the formation of lesions; (10) a method of treating psoriasis; (11) a method of

treating atherosclerosis; (12) a method of treating diseases or conditions involving a plurality of integrin-dependent etiopathogenetic mechanisms; and (13) a method of inhibiting the transfer of genetic material.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graph of HIV-induced syncytium formation (curve 1) or HIV replication (curve 2) as a percentage of untreated control versus concentration of ajoene (micromoles per liter).

For curve 1, the effect of Ajoene on the fusion of cultured, intact H9 cells with HIV infected H9_{RF} cells is disclosed. For this curve, the vertical graph axis pertains to the maximum amount of syncytia formed in the absence of Ajoene (100 percent), while the points on line 1 represent percentages of such an amount of syncytia formed in the presence of varying concentrations of Ajoene (micromoles per liter). The IC₅₀ of syncytium formation was found at a concentration of 0.045 micromole of Ajoene per liter. Essentially no syncytia were found at a concentration of 50 micromoles of Ajoene per liter.

Referring to curve 2, the antiviral activity of Ajoene is shown, as assessed by the inhibition of HIV-1_{Lav-BRU1} replication in cultured CEM13 cells. Here, the vertical axis represents the percentage of HIV antigens detected by solid phase immunoassay in the absence of Ajoene. The IC₅₀ was achieved under these conditions at about a 5 micromolar concentration of Ajoene.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

By this invention, safe and effective doses of Ajoene may inhibit the progression of an HIV

infection in a patient. Specifically, stereoisomer mixtures of Ajoene may be used, although pure (E) and (Z) stereoisomer forms may also be used.

One of the characteristics of an HIV infection so inhibited is the formation of HIV-induced syncytia, in which HIV target cells such as lymphocytes and monocytes fuse together to form giant, multinucleate cells in HIV-infected patients. This inhibiting effect would require a sufficient dose to provide a concentration of Ajoene of at least 50 nanomoles of Ajoene per liter of blood plasma in the patient, and, if a single dose is used, preferably at least 5 micromoles of Ajoene per liter of a patient's blood plasma, although lesser quantities may also suffice. Transfer of genetic material between cells will, thereby, also be inhibited by Ajoene.

Additionally, it appears that Ajoene inhibits the entry of the infective HIV material into its target cells, including CD4-negative cells, both virus-to-cell and cell-to-cell, and the production of HIV and other viruses by the infected cells. For these purposes, Ajoene is preferably administered in a sufficient dose to provide a concentration approaching or exceeding 5 micromoles of patient's blood plasma, although lesser concentrations may also be effective. In the particular case of CD4-negative cells, Ajoene is preferably administered in sufficient dose to provide a concentration approaching or exceeding 200 micromoles of patient's blood plasma, although lesser concentrations may be effective.

In addition to infections caused by HIV and other viruses of the Retroviridae family, Ajoene can be used to treat infections caused by Herpesviridae (e.g., herpes simplex, varicella zoster, Epstein-Barr virus, and cytomegaly virus), Hepadnaviridae (e.g., hepatitis B), Picornaviridae (e.g., hepatitis A virus

and poliomyelitis virus), Orthomyxoviridae (e.g., influenza virus), Poxviridae (e.g., variola virus and vaccinia virus), Flaviviridae (e.g., yellow fever virus and hepatitis C virus), Rubiviridae (e.g., rubella virus), Paramyxoviridae (e.g., measles, parainfluenza, mumps and canine distemper viruses), Rhabdoviridae (e.g., rabies virus), Papovaviridae (e.g., papillomavirus), and Adenoviridae.

Ajoene also serves as an agent that inhibits the adhesion and aggregation of blood cells, such as platelets and neutrophils. Ajoene, therefore, exhibits benefit as an agent for the treatment of pathologies derived from adhesion and aggregation of these and other cells, including various types of inflammation.

Inflammation, a pathological process inherent in a variety of distinct diseases and illnesses, is defensive in nature, but potentially dangerous if uncontrolled. When viewed at the "whole body" level, an inflammation is most frequently characterized by several localized manifestations (indices), including hemodynamic disorders (e.g., hyperemia and edema), pain, temperature increment, and functional lesion. These inflammatory phenomena are underlain by changes at the cellular and molecular levels. At the cellular level, inflammation is characterized by leukocyte extravasation (a process involving adhesion of leukocytes to the endothelium of the vessel wall and migration into tissue where they may phagocytose bacteria, viruses, and cell debris) and platelet aggregation (a mechanism whereby the spread of the infection is prevented). At the molecular level, inflammation is characterized by activation of at least three plasma defense systems (complement, kinin, and coagulation/fibrinolysis cascades) and by synthesis of cytokines and

eicosanoids. When inflammation becomes generalized, as in the case of shock, various indices of inflammation occur systemically throughout the entire organism.

5 In cases of shock, platelets and leukocytes (principally neutrophils) aggregate in the blood vessels, leading to the development of a clinical condition known as multiple organ failure. The primary organ affected in shock patients is commonly
10 the lung. Lung failure, or adult respiratory distress syndrome (ARDS), a destructive inflammation resulting from adhesion, aggregation, and degranulation of activated neutrophils in the pulmonary microvasculature, may be the main cause of death in
15 patients suffering shock. Ajoene may thus counteract at least part of the effects of shock, whether arising, for example, from sepsis, anaphylaxis, or from blood loss. The inhibiting effect of Ajoene in this use would typically be the result of a dose to
20 provide a concentration of Ajoene approaching or exceeding 5 micromoles per liter of the patient's blood plasma, and, in the case of a single dose, preferably approaching or exceeding 100 micromoles per liter of the patient's blood plasma, although lesser
25 concentrations may be effective.

Ajoene can also be administered in effective dosages to suppress many other acute inflammatory processes, such as those associated with peritonitis, meningitis, and ischemia-reperfusion. Ischemia-
30 reperfusion injury occurs in heart, brain, kidney, liver, lung, or intestinal tract when blood supply to these organs is abruptly stopped (ischemia) and then resumed (reperfusion) after a short period.

With the onset of ischemia and the decrease
35 in the perfusion pressure, neutrophils (because they are larger and less deformable than erythrocytes) are

retained in the capillaries. As the ischemia progresses, cytokines (and other chemoattractants) that are released into the capillary lumina increase the adhesiveness of the retained neutrophils to the endothelium and to each other. Aggregates of neutrophils thus formed obstruct postcapillary venules ("no-reflow," or "no-washout") and attenuate the restoration of the blood flow in the affected region, precluding its reoxygenation and extending the area of ischemia. Activated neutrophils trapped in the capillaries release hydrolytic enzymes and reactive oxygen species (i.e., the armamentarium ordinarily used to defend the host against microorganisms), producing a destructive inflammation.

Restoration of the blood flow, however, further augments the severity of the inflammation thus developed. Neutrophils arriving to the previously ischemic region are activated (by chemoattractants and/or products released by the trapped neutrophils) and recruited into the tissue, where the defensive machinery of the cells is once again used against the host (secondary injury). Notably, ischemia-reperfusion produces a more extensive injury than ischemia per se, which accounts for the tissue damage observed in various diseases and states, such as myocardial infarction, intestinal necrosis, and stroke. Ischemia-reperfusion injury can also be generalized, e.g., in the case of resuscitation after hemorrhagic shock (Mazzone, A., et al., Leukocyte CD11/CD18 Integrins: Biological and Clinical Relevance, 80 Haematologica 161, 1995; see also, Reinhart W.H., Hemorheology: Blood Flow Hematology, 125 Schweiz. Med. Wochenschr. 387, 1995).

Ajoene is also a potent inhibitor of adhesive interactions for other cells, such as lymphoid cells. Adhesion of lymphocytes to each other

and nonlymphoid cells is prerequisite to the development of any immune response, including adverse, undesirable, and self-destructive responses. Ajoene may, therefore, serve as an agent for the prevention, treatment, and control of various immunopathologies.

One group of such immunopathologies comprises diseases stemming from divers allergic reactions (e.g., delayed type hypersensitivity, Arthus reaction, and anaphylaxis). Allergy is an anomalous immune response to antigen challenge, characterized by recruitment of specific leukocyte subsets (e.g., cytotoxic lymphocytes and/or eosinophils) to the tissue, resulting in inflammation. Development of allergic inflammation is the main component in the pathogenesis of many diseases and illnesses, including, e.g., asthma, eczema, purpura pigmentosa chronica, various vasculitides, and hay fever, in addition to those mentioned above. Ajoene serves to control these diseases and illnesses.

Allograft rejection is another example of an undesirable immune response, in which the transplanted organ is recognized by the immune system as a foreign body ("non-self") and attacked in sequence by cytotoxic lymphocytes and phagocytes recruited from the circulation (in a manner similar to that described for reperfusion, supra). This inflammatory response results in progressive disruption of the tissue and graft necrosis. Ajoene may be used to prevent cell recruitment into transplanted tissue and thereby prolong graft survival.

Moreover, the transplanted organ also contains lymphocytes, which, in turn, recognize their new environment as "non-self." The immune response initiated by these donor lymphocytes in the body of the recipient produces a condition known as graft-versus-host disease (GVHD), which can lead to injury,

both acute and chronic. Ajoene shows a significant potential in controlling both acute and chronic GVHD.

Self-destructive responses are caused by the failure of the immune system to distinguish "self" from "non-self." This group of immunopathologies comprises a wide variety of diseases (herein termed collectively "autoimmune diseases"), including, without limitation, rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, multiple sclerosis, insulin-dependent diabetes mellitus, crescentic glomerulonephritis, Graves disease, Hashimoto's thyroiditis, and vasculitides. Other conditions and diseases may also fall into this category (see, e.g., the discussion of psoriasis, p. 16, *infra*). In spite of pronounced differences in the clinical picture of the various autoimmune diseases, the underlying mechanisms involve, in every case, recruitment of leukocytes to organs/tissues affected, resulting in destructive inflammation. Ajoene can be used to reduce or prevent this inflammation and thereby suppress the abnormal immune response. Accordingly, ajoene may be used to treat autoimmune diseases.

The beneficial effects of Ajoene are achieved because Ajoene is a modulating agent for integrins. As used herein, "modulate" means to affect the ability of a particular integrin to perform any of its functions. A modulating agent may act on an integrin directly, e.g., by binding to a portion of at least one subunit (α or β) of the integrin. The agent may also act in some other fashion that is not considered direct, e.g., through any of the various cellular substances and structures which ordinarily interact with or enable the target integrin. These substances and structures include, without limitation, transmembrane proteins (e.g., integrins themselves and

integrin-associated proteins), membrane phospholipids, intracellular molecules with messenger-like function (e.g., integrin-modulating factor), enzymes, and regulatory and signaling proteins. Thus, for example, a modulation may result from alteration in integrin conformation, disassociation of the α and β integrin subunits (or any of the parts thereof), disassociation of integrin clusters (and of clusters formed by integrins with other proteins), or from the loss of integrin-cytoskeleton connections, although modulation may also occur from other types of effects. The functions of integrins, as defined herein, are interrelated and include, inter alia, signaling, adhesion, fusion, and internalization.

As a result of its ability to modulate integrins, Ajoene can be used to treat any disease or condition that involves an integrin-mediated function as a mechanism, including those described above. For instance, Ajoene can be used to inhibit virus-cell fusion or undesired cell-cell fusion. Undesired cell-cell fusion can include cell-cell fusion (transitory or permanent) that results in the transfer of viral genetic material and cell-cell fusion that results in the formation of multinucleate cells (e.g., syncytia, giant cells, and osteoclasts). It can also include undesired fertilization of eggs by sperm and the formation of multinucleate germinal cells (syncytiotrophoblast). Thus, Ajoene can be used as a contraceptive, being administered per vaginam (topically), per os, or in any other appropriate way, when used for this purpose.

Ajoene also prevents conception at the stage of embryo implantation. For example, it can prevent the initial adhesion of the blastocyst to the endometrium and the migration of cytotrophoblasts through the maternal epithelium (i.e., processes

similar to certain steps in the leukocyte extravasation cascade). Furthermore, Ajoene is capable of inhibiting cytotrophoblast invasion, a process differing from extravasation in that it goes from the tissues to the vascular lumen and that the invading cells cross the blood-tissue barrier from outside of the vessel. For example, the production of proteolytic enzymes that are used by cytotrophoblasts to penetrate the basement membrane is governed by integrin outside-in signaling. Modulation of the signaling function of integrins by Ajoene can either completely prevent the production of the requisite enzymes or attenuate it to an extent precluding invasion. Thus, Ajoene can be used as an effective emergency contraceptive to prevent unwanted pregnancy or interrupt it at an early stage.

There are yet other mechanisms whereby Ajoene can exert contraceptive effects. For example, it can prevent the chemotactic response of sperm in the vaginal environment (a specific case of cell homing) and sperm interactions with the epithelium of the female genital tract. Moreover, when administered to males, Ajoene can modulate integrins in sperm precursors and other testicular or epididymal cells, thereby interfering with the maturation processes and resulting in the production of fertilization-incompetent gametes or inhibition of fertilization-competent gametes.

The development of major bone diseases, including osteoporosis, is underlain by excessive bone resorption. This fundamental function is performed by osteoclasts. Osteoclasts are unique multinucleate bone cells formed by fusion of mononuclear progenitors called preosteoclasts. The regulation of osteoclast formation may be achieved by agents acting at various levels of osteoclast formation, including

preosteoclast fusion (Zaidi, et al., Cellular Biology of Bone Resorption, 68 Biol. Rev. 197, 1993). Ajoene can regulate bone resorption because it can inhibit the fusion of preosteoclasts necessary for the formation of osteoclasts.

Granulomas are characteristic of chronic inflammatory lesions, such as those found in tuberculosis and other chronic infections. Granulomas are also present in sarcoidosis, a chronic, systemic inflammatory disease of unknown etiology. Granulomas present in cases of chronic infection and in sarcoidosis contain a large number of multinucleate giant cells formed by the fusion of macrophages. Other diseases associated with the formation of multinucleate cells include, without limitation, Crohn's disease, Langerhans cell histiocytosis, and giant cell arteritis. Ajoene can be used to inhibit the formation of these giant multinucleate cells with beneficial therapeutic effects.

Excessive formation of fibrous interstitial tissue (i.e., fibrosis, or sclerosis) is characteristic of certain diseases (such as scleroderma and idiopathic pulmonary fibrosis) and an outcome of chronic inflammatory processes (e.g., glomerular fibrosis). The development of fibrotic lesions and progression of fibrosis, associated with these conditions, diseases, and illnesses, has been linked to abnormal integrin expression and altered cell adhesion patterns. Ajoene can, therefore, be used for treatment of fibrotic lesions.

Lesions observed in skin diseases and illnesses of diverse origin, such as lichen planus, urticaria, dermatofibroma, psoriasiform dermatitides, and keratoses, are characterized by aberrant integrin expression. Ajoene can serve as an agent for the symptomatic treatment of these diseases, being

administered topically, intradermally, and subcutaneously at the site of lesions, or in any other appropriate way, when used for this purpose.

Another disease characterized by the
5 formation of cutaneous lesions is psoriasis. Although the etiology of psoriasis remains to be elucidated, its pathogenesis involves abnormal expression of integrins in target tissue (e.g., in vascular cells, keratinocytes, and dendritic cells), proliferation of
10 endothelial and epidermal cells, and an autoimmune component (recruitment of lymphocytes and macrophages to skin and joints). Ajoene, therefore, can be used to treat psoriasis in multiple respects.

As a result of its antiviral and anti-
15 inflammatory activity, Ajoene is likely to exhibit significant potency in the prevention and treatment of certain diseases with combined etiopathogenesis. As roughly elaborated herein, the term "etiopathogenesis" is used in reference to diseases for which no
20 distinction can be drawn thus far as to the etiology and pathogenesis. One of such diseases is atherosclerosis. Atherosclerosis appears to be associated with a particular type of cytomegalovirus infection characterized by plaque formation along the
25 blood vessels (Melnick, J.L., et al., Cytomegalovirus and Atherosclerosis, 17 BioEssays 899, 1995). A prominent feature of atherosclerosis is the recruitment of monocyte-macrophages into atherosclerotic plaques, which is an integrin-
30 dependent process. Also, proliferation of smooth muscle cells, which contributes to the formation of atherosclerotic lesions, is regulated by integrins. As indicated elsewhere in this document, Ajoene inhibits the transmission of viral infections virus-
35 to-cell and cell-to-cell. Integrin-mediated adhesion and signaling are also inhibited by Ajoene. Thus,

Ajoene, for multiple reasons, can be used to treat diseases involving combinations of integrin-dependent etiopathogenetic factors, including atherosclerosis, both in humans and in other species.

5 Certain neurodegenerative disorders of unclear etiology, e.g., Alzheimer's disease and lateral amyotrophic sclerosis, involve autoimmune inflammation of nervous tissue as a pathogenetic mechanism. Ajoene should, therefore, demonstrate
10 significant potency in mitigating the symptoms of these diseases and slowing down their progression. In particular, studies have shown that other anti-inflammatory treatments benefit Alzheimer's patients (McGeer, P.L., et al., The Inflammatory Response
15 System of Brain: Implications for Therapy of Alzheimer's and Other Neurodegenerative Diseases, 21 Brain Res. Brain Res. Rev. 195, 1995; Breitner, J.C., et al., Delayed Onset of Alzheimer's Disease with Nonsteroidal Anti-Inflammatory and Histamine H2
20 Blocking Drugs, 16 Neurobiol. Aging 523, 1995).

 To treat any one of these conditions, an effective amount of Ajoene is administered. Effective dosage forms, modes of administration, and dosage amounts may be determined empirically, and making such
25 determinations is within the skill of the art. It is understood by those skilled in the art that the dosage amount will vary with the disease or condition to be treated, the severity of the disease or other condition, which integrin(s) is (are) to be modulated,
30 the route of administration, the rate of excretion, the duration of the treatment, the identity of any other drugs being administered, the age, size and species of the animal, and like factors well known in the medical and veterinary arts. In general, a
35 suitable daily dose of Ajoene will be that amount of the compound which is the lowest dose effective to

produce a therapeutic effect. A suggested range of doses is from about 50 nanomoles to about 200 micromoles per liter. However, the total daily dosage will be determined by an attending physician or
5 veterinarian within the scope of sound medical judgment. If desired, the effective daily dose of Ajoene may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day.

10 Ajoene may be administered in any desired and effective manner: as a pharmaceutical preparation for oral ingestion, or for parenteral or other administration in any appropriate manner such as intraperitoneal, subcutaneous, topical, intradermal,
15 inhalation, intrapulmonary, rectal, vaginal, sublingual, intramuscular, intravenous, intraarterial, intrathecal, or intralymphatic. For instance, the topical application of Ajoene to mucous membranes (in the form of creams, gels, suppositories, and other
20 known means of topical administration) can be used to prevent HIV infection of mucosal cells, an important route of HIV transmission. In addition, intralymphatic administration of Ajoene may be advantageous in preventing the spread of HIV within
25 the body. Further, Ajoene may be administered in conjunction with other antiviral drugs, other contraceptives, and other anti-shock or anti-inflammatory drugs or treatments. The Ajoene may be encapsulated or otherwise protected against gastric or
30 other secretions, if desired.

Other modulating agents for cell integrin receptors exhibit similar benefits as Ajoene. Preferred are non-antibody modulating agents for integrins. Particularly preferred are small (low
35 molecular weight), non-antibody modulating agents which are distinct from oligopeptide fragments of

integrin ligands (e.g., ECM proteins, such as fibrinogen and fibronectin) and cyclic derivatives of these fragments.

In particular, disintegrins, and variants and analogs thereof, can be used. Disintegrins are a family of naturally-occurring cysteine-rich peptides originally isolated from viper venom, but also found on cells and elsewhere, many of which contain the sequence Arg Gly Asp (RGD) as an integrin recognition site. Disintegrins are defined by their specific amino acid sequences and three-dimensional structures. "Variants" of disintegrins are disintegrins engineered to have one or more amino acids added, deleted or replaced. "Analogues" are non-peptide mimetics of disintegrins or their variants. Disintegrins and variants thereof, their preparation and properties, are described in numerous publications, as are means of identifying analogs of disintegrins. See, e.g., Sato, et al., 111 J. Cell Biol. 1713, 1990; Rucinski, et al., 1054 Biochim. Biophys. Acta 257, 1990; Gould, et al., 195 Proc. Soc. Exp. Biol. Med. 168, 1990; Kini, et al., 28 Toxicon 1387, 1990; Soszka, et al., 196 Exp. Cell Res. 6, 1991; Calvete, et al., 30 Biochemistry 5225, 1991; Scarborough, et al., 266 J. Biol. Chem. 9359, 1991; Adler, et al., 31 Biochemistry 1031, 1992; Scarborough, et al., 268 J. Biol. Chem. 1058, 1993; Scarborough, et al. 268 J. Biol. Chem. 1066, 1993; Omori-Sato, et al., 24 Toxicon 1045, 1986; Huang, et al., 262 J. Biol. Chem. 16157, 1987; Knudsen, et al., 179 Exp. Cell Res. 42, 1988; Gan, et al., 263 J. Biol. Chem. 19827, 1988; Huang, et al., 28 Biochemistry 661, 1989; Garsky, et al., 86 Proc. Natl. Acad. Sci. U.S.A. 4022, 1989; Shebuski, et al., 264 J. Biol. Chem. 21550, 1989; Chao, et al., 86 Proc. Natl. Acad. Sci. U.S.A. 8050, 1989; Musial, et al., 82 Circulation 261, 1990; Williams, et al., 1039 Biochim.

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- 5 Shebuski, et al., 82 Circulation 169, 1990); Dennis, et al., 87 Proc. Natl. Acad. Sci. U.S.A. 2471, 1990; Mazur, et al., 202 Eur. J. Biochem. 1073, 1991; Saudek, et al., 202 Eur. J. Biochem. 329, 1991; Huang, et al., 109 J. Biochem. 328, 1991; Adler, et al., 253
- 10 Science 445, 1991; Saudek, et al., 30 Biochemistry 7369, 1991; Huang, et al., 1074 Biochim. Biophys. Acta 144, 1991; Huang, et al., 1074 Biochim. Biophys. Acta 136, 1991; Huang, et al., 277 Biochem J. 351, 1991; Huang, et al., 42 Biochem. Pharmacol. 1209, 1991;
- 15 Yamakawa, et al., 109 J. Biochem., 667, 1991; Dalvit, et al., 202 Eur. J. Biochem. 315, 1991; Cooke, et al., 202 Eur. J. Biochem. 323, 1991; Au, et al., 181 Biochem. Biophys. Res. Commun. 585, 1991; Chen,, et al., 30 Biochemistry 11625, 1991; Kini, et al., 30
- 20 Toxicon 265, 1992; Hite,, et al., 31 Biochemistry 6203, 1992; Calvete, et al., 309 FEBS Letters 316, 1992; Sheu, et al., 83 Jpn. J. Cancer Res. 885, 1992; Paine, et al., 267 J. Biol. Chem. 22869, 1992; Takeya, et al., 267 J. Biol. Chem. 14109, 1992; Chang, et al.,
- 25 190 Biochem. Biophys. Res. Commun. 242, 1993; Takeya, et al., 113 J. Biochem. 473, 1993; Adler, et al., 32 Biochemistry, 282, 1993; Dennis, et al., 15 Proteins: Structure, Function and Genetics 312, 1993; Hite, et al., 308 Arch. Biochem. Biophys. 182, 1994.

30 The disintegrins, and variants and analogs thereof, like Ajoene, can be used to treat viral infections, shock, and inflammation, to inhibit fertilization of eggs by sperm, to suppress immune responses, to treat arthritis and autoimmune diseases,

35 to prevent undesired integrin-mediated cell-cell fusion, and to treat other diseases and conditions

against which Ajoene is effective. The disintegrins and variants and analogs thereof may be used for these purposes as described herein for Ajoene. In particular, effective dosage forms, modes of administration and dosage amounts for administration of disintegrins, and variants and analogs thereof, to patients may be determined empirically as described herein for Ajoene, and making such determinations is within the skill of the art. A suggested range of doses is 10 to 5000 nanomoles per liter blood plasma, depending on the condition to be treated, on which disintegrin (or disintegrin analog or variant) is to be used, which integrin(s) is (are) intended to be modulated, and on other pertinent factors, as herein discussed. However, appropriate doses may be derived for any particular condition and mode of administration, based on the relative potency exhibited by Ajoene and disintegrins (or variants or analogs of disintegrin in question) under comparable experimental conditions.

Other suitable integrin modulating agents for use in practice of the invention may be identified by their ability to interfere with integrin-mediated functions. As noted above, fundamental integrin-mediated functions include cell signaling, cell adhesion, cell fusion, and internalization. Simple in vitro screening assays can be used to ascertain if compounds inhibit such integrin-mediated functions. Suitable assays are described in the examples and others are well-known in the art. For instance, in vitro induced platelet aggregation, which is routinely performed in any clinical laboratory, provides a particularly well-known example of a system that allows to detect and measure integrin signaling (inside-out) and integrin-mediated adhesion (see, e.g., Huang, T.-F., et al., Mechanism of Action of the

Antiplaquet Peptide, Arietin, from Bitis arietans
Venom, 1074 Biochim. Biophys. Acta 144, 1991).
Analogous screening systems for neutrophil integrins
have been described (see, e.g., Hoffstein, S.T., et
5 al., Degranulation, Membrane Addition, and Shape
Change During Chemotactic Factor-Induced Aggregation
of Human Neutrophils, 95 J. Cell Biol. 34, 1982).
Similar assays exist for integrin-mediated fusion
(Bronson, R.A., et al., Evidence that an Arg-Gly-Asp
10 Adhesion Sequence Plays a Role in Mammalian
Fertilization, 43 Biol. Reprod. 1019, 1990), integrin-
mediated outside-in signaling (see, e.g., Shattock,
R.J., et al., Cellular Adherence Enhances HIV
Replication in Monocytic Cells, 145 Res. Virol. 139,
15 1995), and integrin-mediated internalization (see,
e.g., Handagama, P., et al., supra; compare, e.g.,
Wencel-Drake, J.D., et al. Arg-Gly-Asp-dependent
Occupancy of GPIIb/IIIa by Applagin: Evidence for
Internalization and Cycling of a Platelet Integrin, 81
20 Blood 62, 1993, demonstrating the existence of a
separate, signal-independent GPIIb/IIIa
internalization pathway).

The above disclosure and the examples below
are for illustrative purposes only, and are not
25 intended to limit the invention of this application,
which is as defined in the claims below.

EXAMPLES

30 Example 1

Ajoene Affects Cellular Activities Mediated by Several Distinct Integrin Receptors

Platelet aggregation was measured
turbidimetrically on a standard platelet aggregometer
35 equipped with a recorder. Samples of platelet-rich
plasma (PRP) were standardized with respect to the

cell density (3×10^8 per ml); maximal light transmission (LT) was calibrated with platelet-poor plasma (PPP). Aliquots of PRP (0.25 ml in glass cuvettes) were stirred in the cell of the device (1000 rpm, 37°C) with Ajoene (0-100 micromoles per liter final concentration) for 1 min., followed by addition of the aggregation stimuli. (Unless specified otherwise, all the experiments described in this Application were performed with Ajoene that represented a 1:1 mixture of E- and Z-stereoisomers.) LT increments were traced for 5 min. Maximal rate of aggregation was derived from the slope of each curve; IC_{50} for Ajoene was determined by a method such as that described in Vrzheschch, et al., Cell Response Kinetics: the Phenomenon of Supercooperativity in Aggregation of Human Platelets, 66 Thrombos. Res. 537, 1992.

Neutrophil aggregation was assayed as described above for the platelet system, with some modifications. Freshly isolated neutrophils were finally suspended in a buffer (120 mM NaCl, 4 mM KCl, 10 mM dextrose, 2 mM $CaCl_2 \cdot 2H_2O$, 2 mM $MgCl_2 \cdot 6H_2O$, and 20 mM Tris; pH 7.4) to the final density of 10^7 cells per ml. Aliquots of neutrophil suspension (0.25 ml in siliconized glass cuvettes; 10^7 cells per ml) were stirred in the cell of the aggregometer with 5 micrograms per ml cytochalasin B (1000 rpm, 37°C) for 1 min. Ajoene (0-50 micromoles per liter) and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (10^{-7} M) were then introduced into the suspension sequentially, with an interval of 1 min. Maximum LT (100%) was calibrated with the same buffer.

T-lymphocytic H9 and Jurkat cells were maintained as suspension cultures in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 micrograms/ml gentamycin and 2 mM L-

glutamine. To assess the effects of Ajoene on adhesive interactions of the cells, H9 cells or Jurkat cells were seeded in 96-well flat-bottomed plates (3×10^5 cells per well) and cultured in the presence of log
5 10 dilutions of Ajoene (experiment) or its vehicle (control) for 16 to 24 h.

Ajoene inhibited platelet aggregation in PRP with an IC_{50} of about 50 micromoles per liter. Ajoene blocked N-formyl-L-methionyl-L-leucyl-L-phenylalanine-
10 stimulated neutrophil aggregation (IC_{100} of about 10 micromoles per liter) and caused rapid deaggregation when added to aggregated neutrophils. The clusters normally formed by H9 cells and Jurkat cells in culture were disrupted by Ajoene with an IC_{50} of about
15 50 nanomoles per liter for each cell system.

The requirement of integrins for the formation of cell-to-cell contacts in each of these systems has long been established. It is known that in platelets the relevant integrin is GP IIb/IIIa
20 ($\beta 3$). Neutrophil aggregation is underlain by interactions of $\beta 2$ integrins (e.g., LFA-1 and/or Mac-1) with their surface counterstructures, while cohesion of T-cells is supported by both $\beta 2$ integrins and fibronectin-binding $\beta 1$ integrins (e.g., VLA-4 and
25 VLA-5). Hence, Ajoene is capable of modulating integrin receptors of at least three distinct subfamilies ($\beta 1$, $\beta 2$, and $\beta 3$).

This example also demonstrates that Ajoene affects at least two integrin functions: inside-out
30 signaling (upregulation of integrins upon platelet and neutrophil activation) and adhesion.

Example 2Integrin-Modulating Properties
of Ajoene are not Stereospecific

Synthetic stereoisomers of Ajoene exhibited
5 equal antiaggregatory activity (IC_{50} of about 50
micromolar concentration of Ajoene for platelets, IC_{100}
of about 10 micromolar concentration of Ajoene for
neutrophils).

10

Example 3Ability Of Ajoene To Modulate Adhesion
of Cells to Substrata, Mediated by VLA-4

Microtiter plates were coated with ligands
of the integrin VLA-4, i.e., recombinant human VCAM-1
15 (500 nanograms per well) or the fragment of connective
segment of human fibronectin (CS-1), covalently
attached to ovalbumin (5 micrograms per well) by
overnight incubation (4°C) with solutions of the
proteins in phosphate-buffered saline (PBS). The
20 plates were blocked with 1% bovine serum albumin (BSA)
in Hanks' buffered salt solution (HBSS-BSA). The
cells (Jurkat or human melanoma MV3) were labeled with
the fluorescent dye 5-chloromethylfluorescein
diacetate (CMFDA) and introduced into the wells of the
25 plates (10^5 cells per well) together with varying
amounts of Ajoene. After incubation at 37°C for 1 h,
the unbound cells were removed by aspiration and the
wells washed four times with HBSS-BSA. Thereafter,
the bound cells were lysed with 0.5% Triton X-100, and
30 the fluorescence was measured using a Cytofluor 2300
plate reader. The number of adherent cells was
calculated from a standard dilution curve of Triton X-
100 lysates of the CMFDA-labeled cells.

Ajoene inhibited VLA-4-dependent adhesion of
35 Jurkat and MV3 cells to VCAM-1 with IC_{50} values of 250
and 125 micromoles per liter, respectively. However,

no inhibition of adhesion to CS-1 could be detected in this concentration range. Therefore, the CS-1 recognition site of VLA-4 was more resistant to modulation with Ajoene than the VCAM-1 recognition site.

Because there is no way for the cells tested to adhere to the substrata otherwise than via VLA-4, this example provides conclusive evidence that Ajoene modulates VLA-4 and affects its adhesion function.

Example 4

Ability of Ajoene to Modulate LFA-1-mediated Homotypic Adhesion and Inside-out Signaling

Normal human peripheral blood leukocytes (PBLs) were cultured for 5 days without stimulation in complete RPMI medium (Sigma Chemical Co., St. Louis, MO), washed, and resuspended at 9×10^6 cells/ml in the same medium. Fifty microliters of the cells were added in duplicate to wells containing 100 microliters of an Ajoene solution (50 micrograms/ml), anti-LFA-1 antibody (H52) solution (20 micrograms/ml), or vehicle controls. The plate was then incubated for 30 min at 37°C before adding to each well 50 microliters of a solution of 12-O-tetradecanoyl 4- β -phorbol 13-acetate (TPA) to the final concentration of 125 ng/ml. The plate was incubated for 45 min at 37°C, followed by additional 10-min incubation at room temperature on a shaking platform. The cells were allowed to settle, and adhesion was quantitated by counting the number of free cells in random high-power fields in the duplicate wells. Following incubation with TPA, 75 to 90% of cells are typically found in large aggregates as a result of LFA-1 upregulation.

TPA induced a 90% aggregation in PBLs that were preincubated with vehicle controls. The aggregatory response was completely inhibited by H52,

the monoclonal antibody used as a positive control, indicating that the aggregatory response was mediated by LFA-1. Pretreatment with Ajoene at ~100 micromoles per liter concentration completely inhibited the TPA-induced homotypic adhesion of PBLs.

This example provides conclusive evidence that, in addition to GP IIb/IIIa and VLA-4, Ajoene modulates the integrin LFA-1. In addition, because TPA-induced homotypic aggregation of PBLs involves LFA-1 upregulation (inside-out signaling), the above result further confirms that modulation of LFA-1 with Ajoene affects both the signaling and adhesion function of this integrin.

Example 5

Ajoene Modulates VLA-4- and LFA-1-mediated Heterotypic Cell Adhesion and Inside-out Signaling

SVT2 cells transfected with human ICAM-1 or VCAM-1 (SVT2/ICAM-1 and SVT2/VCAM-2, respectively) were plated into 96-well plates and cultured overnight. (SVT2/ICAM-1 and SVT2/VCAM-2 were demonstrated previously to express ICAM-1 and VCAM-1, respectively, on their surface.) Jurkat cells were washed and cultured overnight in the presence of 100 microCurie/ml of $\text{Na}_2^{51}\text{CrO}_4$. The labeled Jurkat cells were washed three times and resuspended at a density of 4×10^6 cells/ml in complete RPMI medium. Serial dilutions of Ajoene were prepared in complete RPMI medium. Aliquots of the cells (0.5 ml) were then mixed with an equal volume of Ajoene dilutions (1:1 v/v), and the mixtures incubated at 37°C for 30 min. Thereafter, Ajoene-incubated cells were added in duplicate to SVT2/ICAM-1 and SVT2/VCAM-1 monolayers, and TPA was introduced into each well to the final concentration of 125 ng/ml. The plates were incubated at 37°C for 45 min, after which the non-adherent cells

were removed by washing with warm complete RPMI medium. The bound labeled cells were lysed by adding 1% Triton X-100 solution in 0.1 N HCl. The lysates were assayed in a gamma counter to determine the percentage of bound cells.

Because SVT2 cells do not contain the ligands for LFA-1 and VLA-4, adhesion to Jurkat cells becomes possible only after transfection of SVT2 cells with ICAM-1 or VCAM-1. Thus, coculturing of Jurkat cells with SVT2/ICAM-1 and SVT2/VCAM-1 cells makes it possible to assess, respectively, the function of LFA-1 and VLA-4 under identical conditions. In the absence of TPA, 10 and 20% of the added Jurkat cells adhered to SVT2/ICAM-1 and SVT2/VCAM-1 cells, respectively. Activation of Jurkat cells by TPA and the resulting upregulation of LFA-1 and VLA-4 (inside-out signaling) invariably produced adhesion levels in excess of 90% in both experimental settings. Ajoene inhibited adhesion of activated Jurkat cells to SVT2/ICAM-1 and SVT2/VCAM-1 monolayers with IC₅₀ values of 10 micromoles per liter and ~20 micromoles per liter, respectively. This difference in the relative potency of inhibition was statistically significant, indicating that LFA-1 is slightly more sensitive to Ajoene than VLA-4.

This example demonstrates that Ajoene modulates LFA-1 and VLA-4 and impairs the inside-out signaling and adhesion functions of these integrins.

Example 6

In Vivo Modulation by Ajoene of LFA-1 and
VLA-4-mediated Recruitment of
Monocytes and Neutrophils into Inflamed Joints

5 Rat adjuvant arthritis is characterized by
integrin-mediated migration of ⁵¹Cr-labeled monocytes
and ¹¹¹In-labeled neutrophils into the inflamed
synovium, which attains maximum between days 14 and 21
after the immunization. This model of human
10 rheumatoid arthritis was used to assess *in vivo*
effects of Ajoene on integrins that are known to be
modulated by Ajoene *in vitro*.

 Adjuvant arthritis was induced in 6-8 week
old inbred male Lewis rats by immunization on the
15 lower back with 0.5 mg *Mycobacterium butyricum* (Difco
Laboratories, Inc., Detroit, MI) in 0.05 ml mineral
oil in two sites subcutaneously at the base of the
tail. All the animals developed polyarticular
arthritis between days 11 and 13 after immunization.
20 For clinical scoring, 0-4 points were assigned per
limb and tail, based on the severity of erythema,
swelling, and limitation of movement (the maximal
possible score was 20).

 Rat blood monocytes and neutrophils for
25 migration studies were obtained by the hydroxyethyl
starch (HES) exchange transfusion technique. The
total blood volume of a donor rat was exchanged using
50 ml 6% HES solution in saline (DuPont Chemical Co.,
Dorval, Canada). After recovery of 40-45 ml blood/HES
30 perfusate, the red cells were allowed to sediment (1
g) and the leukocyte-rich plasma was harvested. The
leukocytes were recovered by centrifugation,
resuspended in calcium-magnesium-free Tyrode's
solution supplemented with 10% platelet-poor plasma
35 (TS-PPP), and centrifuged in a discontinuous 63%/74%
gradient of isotonic Percoll (Pharmacia Fine
Chemicals, Dorval, Canada). The top layer, containing

mononuclear cells (MN), and the cells in the 63%/74% interface, containing neutrophils, were collected, washed, and resuspended in TS-PPP. To obtain purified monocytes, the osmolality of the MN suspension was
5 increased by adding 9% NaCl, and the suspension was centrifuged in a 40%/55%/58% Percoll gradient; monocytes were harvested at 40%/55% and 55%/58% interfaces, washed, and resuspended in TS-PPP.

Thereafter, monocytes (10^7 cells/ml) were
10 labeled with 75 microCurie/ml of $\text{Na}_2^{51}\text{CrO}_4$ (Amersham, Oakville, Canada) at 37°C for 30 min. Neutrophils (10^7 cells/ml) were labeled with 1 microCurie/ml of ^{111}In oxine (Amersham) for 10 min. Both populations were washed to remove excess labeled material and checked
15 for viability and purity. The viability of either cell population used in the experiments was in excess of 95% (as double-checked by Trypan blue exclusion and neutral red staining). The separation procedure described above ordinarily produced populations that
20 contained no less than 90% monocytes and no less than 95% neutrophils.

Ajoene was administered to anaesthetized arthritic rats intravenously (50 mg/kg per rat; infusion rate, 5 mg/min), as a suspension in
25 Intralipid, a parenteral nutritive containing fractionated soybean oil (200 g/l), fractionated egg phospholipids (12 g/l), and glycerol (22 g/l). To prepare this suspension, Ajoene was first dissolved in ethanol to the concentration of 2 moles per liter.
30 This solution was then introduced under vigorous stirring into Intralipid (KabiVitrum; Stockholm, Sweden) to the final concentration of 0.085 moles per liter (20 mg/ml) Ajoene; the content of ethanol in the suspension thus obtained did not exceed 3% (v/v).
35 Control arthritic rats received, under the same experimental conditions, adequate volumes of the

vehicle (Intralipid supplemented with 3% ethanol v/v). All subsequent manipulations with animals of both groups were identical; on the day of the experiment, mean scores in control and treatment rats were 12.5 and 14.0, respectively.

Within 2-3 min of Ajoene administration, each rat was injected intravenously with ^{51}Cr -labeled monocytes ($4-6 \times 10^6$ monocytes, carrying $1.0-1.5 \times 10^5$ cpm ^{51}Cr) and ^{111}In -labeled neutrophils (10^7 neutrophils, carrying $2-5 \times 10^5$ cpm ^{111}In). After 2 h, 1 ml of blood was collected from each rat (for differential leukocyte counts and plasma ^{51}Cr) and the animals were sacrificed. The hindlimbs were sectioned just above and below the tibiotalar joint, providing tarsal and talar joint samples for gamma counting (model 1280 gamma spectrometer; LKB Instruments, Inc.; Gaithersburg, MD). The activity of the tail was taken as a measure of the process in vertebral joints.

The results are summarized in the tables below.

Table 1. Inhibition by Ajoene of monocyte recruitment to inflamed joints

Sample	Accumulation of ^{51}Cr -labeled Monocytes, cpm per sample (mean \pm SEM)		Significance
	Control	Ajoene	
Talar joint	42,304 \pm 3,618	25,078 \pm 2,872	$P < 0.006$
Tarsal joint	16,604 \pm 4,374	10,365 \pm 2,572	N.S.
Vertebral joints	5,262 \pm 1,400	2,737 \pm 521	$P = 0.079$

Table 2. Inhibition by Ajoene of neutrophil recruitment to inflamed joints

Sample	Accumulation of ^{111}In -labeled Neutrophils, cpm per sample (mean \pm SEM)		Significance
	Control	Ajoene	
Talar joint	31,084 \pm 1,940	15,052 \pm 1,570	$P < 0.0001$
Tarsal joint	12,554 \pm 2,534	7,353 \pm 1,438	$P = 0.085$
Vertebral joints	5,775 \pm 782	3,658 \pm 546	$P = 0.089$

Treatment with Ajoene resulted in a highly significant, ~50% inhibition of monocyte (Table 1) and neutrophil (Table 2) recruitment to the talar joints, which are the most susceptible to inflammation in this model. Moreover, these joints yield consistent recruitment of monocytes and neutrophils, and, therefore, the values obtained are the most reliable. (The tarsal joints and vertebral spine joints are also affected by the inflammation, but the variability is greater, than in the case of talar joints, and, consequently, the effects of treatment with Ajoene are not as pronounced.)

Notably, blocking monoclonal antibodies (MABs) against VLA-4, LFA-1, or CR3 do not inhibit monocyte or neutrophil accumulation in the talar joints, when given alone. Only combined administration of antibodies against different integrins (e.g., anti-LFA-1 plus anti-VLA-4 or anti-LFA-1 plus anti-Mac-1; see, Issekutz, A.C., et al., Monocyte Migration to Arthritis in the Rat Utilizes both CD11/CD18 and Very Late Activation Antigen 4 Integrin Mechanisms, 181 *J. Exp. Med.* 1197, 1995, and Issekutz, T.B., et al., Rat Blood Neutrophils Express Very Late Antigen 4 and It Mediates Migration to

Arthritic Joint and Dermal Inflammation, 183 *J. Exp. Med.* 2175, 1996) produces an inhibition that is comparable in extent with that induced by Ajoene.

Thus, Ajoene exerts *in vivo* the same
5 integrin-modulating effects as *in vitro*, i.e., it acts on several distinct integrins. In particular, this example demonstrates a pronounced *in vivo* effect on $\beta 2$ integrins (e.g., LFA-1) and $\beta 1$ integrins (e.g., VLA-4), although modulation of other integrins cannot be
10 ruled out.

In vivo inhibition by Ajoene of leukocyte recruitment in a model of rheumatoid arthritis indicates that Ajoene can be used to treat rheumatoid arthritis, as well as other autoimmune (e.g., lupus,
15 diabetes, Sjögren's syndrome, and vasculitides) and inflammatory diseases (e.g., ischemia-reperfusion injury and graft rejection) characterized by integrin-mediated recruitment of leukocytes. Also, other types of cell migration, which are critical to the
20 development of undesirable conditions, diseases, and illnesses (e.g., embryo implantation) will likewise be inhibited by Ajoene *in vivo*.

Example 7

25 *In Vivo* Modulation by Ajoene of LFA-1- and VLA-4 -mediated Recruitment of Monocytes and Neutrophils into Inflamed Skin

Chemotactic factors (e.g., C5a_{desArg}) and cytokines, such as tumor necrosis factor α (TNF) or
30 interferon gamma (IFN), induce rapid cutaneous inflammatory reactions, when injected intradermally. These inflammatory reactions are characterized by integrin-mediated migration of ^{51}Cr -labeled monocytes and ^{111}In -labeled neutrophils. The mechanisms of
35 leukocyte recruitment to the inflammatory loci differ, however, in that chemotactic factors act primarily on leukocytes (by upregulating integrins), whereas TNF

and IFN activate endothelial cells (which results, *inter alia*, in rapid expression of integrin ligands, such as ICAM-1 and VCAM-1).

Agents used for the induction of the
5 inflammation included rat IFN, recombinant mouse TNF (Genentech, South San Francisco, CA), and Zymozan-activated serum (ZAS). ZAS, a source of C5a_{desArg}, was generated by activating complement in normal rat serum with 5 mg/ml of Zymozan A (Sigma Chemical Company, St.
10 Louis, MO) at 37°C for 60 min, after which the zymozan was removed.

Rat monocytes and neutrophils were isolated and labeled as described in Example 6, *supra*. Male Lewis rats were administered intravenously with 50
15 mg/kg Ajoene (treatment group) or its vehicle (control), followed within 2-3 min by infusion of the labeled cells. Immediately afterwards, the skin on the back of all animals was shaved and the inflammatory stimuli and the diluent control were
20 injected intradermally at several sites of the skin of each animal. Two hours later, 1 ml of blood was taken from each rat, and the animals were sacrificed. The dorsal skin, including the area of dermal inflammatory reactions, was removed and frozen, and the injected
25 skin sites were punched out with a 12-mm punch and counted in a gamma spectrometer (model 1280, LKB Instruments).

Table 3. Inhibition by Ajoene of monocyte recruitment to inflamed skin

5

Inflammatory stimulus	Accumulation of ⁵¹ Cr-labeled Monocytes, cpm per inflammatory locus (mean ± SEM)		Significance
	Control	Ajoene	
50% ZAS	2,550 ± 274	1,268 ± 170	<i>P</i> < 0.002
25% ZAS	1,557 ± 158	944 ± 127	<i>P</i> < 0.020
10 ng TNF	2,506 ± 70	1,274 ± 140	<i>P</i> < 0.002
300 U IFN	1,747 ± 205	468 ± 64	<i>P</i> < 0.001
Diluent control	82 ± 23	15 ± 10	N.S.

10

15

Table 4. Inhibition by Ajoene of neutrophil recruitment to inflamed skin

20

Inflammatory stimulus	Accumulation of ¹¹¹ In-labeled Neutrophils, cpm per inflammatory locus (mean ± SEM)		Significance
	Control	Ajoene	
50% ZAS	11,486 ± 776	7,926 ± 378	<i>P</i> < 0.001
25% ZAS	7,751 ± 280	5,558 ± 236	<i>P</i> < 0.001
10 ng TNF	1,750 ± 36	1,289 ± 84	<i>P</i> < 0.006
300 U IFN	223 ± 42	160 ± 11	<i>P</i> < 0.060
Diluent control	62 ± 13	53 ± 8	N.S.

25

Treatment with Ajoene resulted in a highly significant inhibition of monocyte migration to loci of acute cutaneous inflammation (by 50-75%). In the case of neutrophils, the inhibition was less pronounced (by 30-40%), but also statistically significant. None of the anti-integrin MABs tested previously in the same model inhibited the cell

30

recruitment to any significant degree, when administered alone. Only a combination of an anti-LFA-1 and an anti-VLA-4 MABs demonstrated the same degree of attenuation of monocyte/neutrophil accumulation at the loci of cutaneous inflammation as Ajoene.

This example provides further evidence that Ajoene modulates *in vivo* $\beta 2$ and $\beta 1$ integrins, e.g., LFA-1 and VLA-4, although effects on other integrins are also likely to occur. The *in vivo* integrin modulation by Ajoene results in significant inhibition of leukocyte recruitment to inflammatory loci and attenuation of inflammation. Notably, the anti-inflammatory activity of Ajoene shows essentially no dependence on the primary mechanism operative in the recruitment of leukocytes to the inflammatory loci (e.g., chemotactic stimulation of leukocytes vs. cytokine activation of the endothelium).

Thus, this example indicates that Ajoene can treat a broad range of inflammatory diseases, regardless of the particular mechanism of inflammation. More specifically, this example underscores the ability of Ajoene to treat various skin diseases and illnesses, such as psoriasis, scleroderma, eczema, and allergy.

When viewed together, Examples 6 and 7 provide significant evidence that *in vivo* modulation of integrins by Ajoene takes place in various locations of the body. Whatever organ or tissue is affected, Ajoene will modulate its target integrins in essentially the same way as it modulates them *in vitro*. Thus, Ajoene can treat diseases involving multiple locations or occurring systemically throughout the whole body (e.g., shock and generalized ischemia-reperfusion injury).

Example 8Ajoene as an Inhibitor of Fusion Leading to Syncytium Formation in HIV-infected Cells

Chronically infected and uninfected cell lines (HIV donors and targets, respectively) were maintained as suspension cultures in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 micrograms/ml gentamycin and 2 mM L-glutamine. HIV donors used were (1) a cloned population of H9 cells harboring the genome of HIV-1_{RF} (H9_{RF} cells); and (2) a cloned population of SupT1 cells harboring the genome of HIV-1_{LAV-BRU} (SupT1_{LAV-BRU} cells). To assess the effect of Ajoene on syncytium formation, the donors were mixed with their respective recipients, H9 and SupT1, at a ratio of 1:2 in 96-well flat-bottomed plates (3 x 10⁵ cells per well). Log 10 dilutions of Ajoene were then introduced into the wells, and the cells were cocultured for 16 h. Control wells received the vehicle of Ajoene. By the end of the incubation period syncytia were counted, and the values of IC₅₀ for Ajoene were calculated by plotting relative amounts of syncytia (percent of the syncytia formed in the absence of the compound) against Ajoene concentration.

Table 5.

HIV donor	HIV recipient	IC ₅₀ of Ajoene, nanomoles/l
H9 _{RF}	H9	~ 50
SupT1 _{LAV-BRU}	SupT1	~ 5

Specifically, Ajoene inhibited the fusion of uninfected cells with HIV-infected cells and the formation of syncytia, resulting from this fusion, with IC₅₀ values in the range from 5 to 50 nanomoles per liter Ajoene concentration at 16 h incubation.

The fusion machinery of cells operative in the formation of syncytia in HIV infection involves as components $\beta 1$ and $\beta 2$ integrins (e.g., VLA-3 and LFA-1). Thus, this example demonstrates that modulation
5 by Ajoene of $\beta 1$ and $\beta 2$ integrins affects yet another fundamental integrin function, fusion.

Taken together with the examples provided *supra*, this example provides conclusive evidence that Ajoene will inhibit the formation of syncytia
10 associated with HIV infection in patients. Furthermore, fusion events associated with other viral infections, noninfectious diseases (e.g., granulomatous inflammations and osteoporosis), or undesirable conditions (e.g., pregnancy) can also be
15 prevented, attenuated, or inhibited by appropriate *in vivo* administration of Ajoene.

Example 9

Ajoene Inhibits HIV Replication by Modulating 20 Integrin-mediated Outside-in Signaling

LAV-BRU 1 strain of HIV-1 and RF strain of HIV-1 were propagated in CEM and H9 cells, respectively. A 20-ml log-phase culture containing 7×10^5 cells per ml was incubated at 37°C for 24 h.
25 Cultures with less than 10% Trypan blue stained cells were used as a source of virus. To harvest HIV particles, producer cells were pelleted by centrifugation (400 g, 5 min) and the supernatant containing HIV-1 particles was filtered through a 0.45
30 micrometer Millipore filter, aliquoted, and stored at -80°C. The titer of HIV-1 ranged from 1×10^5 to 2×10^5 CCID₅₀ (50% cell culture infective dose). To determine whether Ajoene could influence HIV replication, CEM13 and H9 cells were inoculated with
35 appropriate amounts of HIV (LAV-BRU 1 and RF, respectively), to give an m.o.i. (multiplicity of

infection) of 0.1 in either experimental setting. Ajoene (or its vehicle) was added to 10^7 infected cells resuspended in the growth medium and the cells were plated in 96-well flat-bottomed plates. After 72 h the bulk of HIV antigens was measured by solid phase immunoassay as described in Zhdanov, V.M., et al., An Enzyme Immunoassay for Detection of the Antigen and Antibody to the Human Immunodeficiency Virus and the Use Thereof For Serological Survey of Different Population Groups, 33 Vop. Virusol. 294, 1988 (in Russian). In a series of separate experiments Ajoene was introduced into plated cell cultures stepwise, i.e. in aliquots of 50 nanomoles per liter concentration per 12 h of incubation, the first addition coinciding with the moment of inoculation.

Replication of HIV-1_{RF} in H9 cells was inhibited with an IC₅₀ of 25 micromoles per liter (m.o.i. 0.1; 72 h of incubation). Assessment of HIV-1_{LAV-BRU 1} replication in CEM13 cells under the same experimental conditions revealed a more pronounced antiviral activity (IC₅₀ of about 5 micromoles per liter concentration). A considerable increase in the antiviral activity became evident when the concentration of the compound was increased stepwise (50 nanomoles per liter concentration per 12 h of incubation; CEM13 - LAV-BRU 1 system; inhibition by 30%; total concentration 0.25 micromoles per liter; m.o.i. 0.1; 72 h of incubation).

This example demonstrates that Ajoene affects the outside-in signaling function of integrins, thereby preventing the replication of HIV. The replication of HIV and of other viruses involves, as an essential step, the use of host cell protein-synthesizing machinery, which is regulated by integrin-elicited signals. Modulation of the outside-in signaling via integrins is, therefore, a way to

control viral genes and the synthesis of viral proteins by the infected cells. Thus, administration of Ajoene to patients with viral infections can block the production of viral material and prevent the spread of viruses in the infected individual.

Furthermore, other cellular functions, not related to viral infection, can also be controlled by Ajoene, as this example demonstrates. These cellular functions include, without limitation, cell division, cell differentiation, and cell metabolic activity, such as production of mediators and enzymes. In the case of shock, for example, the synthesis of TNF depends on the outside-in signaling function of LFA-1 and can be prevented by appropriate modulation of this integrin (see, Watanabe, S., et al., Prevention of Endotoxin Shock by an Antibody against Leukocyte Integrin $\beta 2$ through Inhibiting Production and Action of TNF, 7 *Int. Immunol.* 1037, 1995; Mukaida, N., et al., Novel Insight into Molecular Mechanism of Endotoxin Shock: Biochemical Analysis of LPS Receptor Signaling in a Cell-Free System Targeting NF- κ B and Regulation of Cytokine Production/Action through $\beta 2$ Integrin *in Vivo*, 59 *J. Leuk. Biol.* 145, 1996.). In a similar way, modulation of integrins and of their outside-in signaling function blocks the production of metalloproteinases, enzymes necessary for the invasion of trophoblasts (Loke, Y.W., et al., Adhesion Molecules and Trophoblastic Invasion, 23 *Contracept. Fertil. Sex* 573, 1995). Thus, this example provides further evidence of the efficacy of Ajoene against a wide range of diseases and undesirable conditions.

Example 10

Ability Of Ajoene To Protect
Cells From Infection With Free Viruses and
Virus-associated Pathologies

5 The effect of Ajoene in protecting against
HIV infection was evaluated using polymerase chain
reaction (PCR) analysis which recognized amplified DNA
sequences of HIV gag gene. MT-4 cells were used as
target cells and strain IIIB of HIV-1 was used as
10 inoculate. Serial dilutions of Ajoene or an
equivalent volume of its vehicle were added to MT-4
cells, followed by addition of a sufficient amount of
free virus ($\geq 10^3$ particles per cell). The protective
effect of Ajoene was monitored by examining for the
15 absence or presence of proviral HIV DNA in target
cells 16 hours after the inoculation. Ajoene
completely prevented HIV infection at the minimal dose
of 4 mg/ml (17 micromoles per liter) and had an IC_{50} of
5 micromoles per liter, without any toxic effects on
20 the cells.

 The effect on herpes simplex virus (HSV)
infection was assessed using human embryonic lung
fibroblast line WI-38 and HSV-2 strain G (derived from
a human with the genital infection). Semiconfluent
25 fibroblast monolayers grown in 96-well culture plates
(10^5 cells per well) were exposed to serial dilutions
of Ajoene or vehicle control, followed immediately by
addition of HSV-2 (5000 CCID₅₀ per well). After 24
hours of culturing, the supernatants were examined by
30 ELISA for the content of HSV antigens. Ajoene
completely prevented HSV infection at 4 mg/ml (17
micromoles per liter) and had an IC_{50} of 6 micromoles
per liter, again, with no detectable toxicity.

 This example provides evidence of Ajoene
35 efficacy against the transmission of viral infections
by free viruses. This mode may play a role in the

transmission of the viruses both from infected individuals to uninfected individuals and from infected to intact cells. Thus, Ajoene can prevent both the entry of various viruses into the human body and the spread of viral infections within the infected individual's body.

In addition, as this example indicates, Ajoene can be used as an effective agent blocking sexual transmission of HIV and HSV. In addition, the entry of other microorganisms known to use the same portal to invade the human body will likewise be prevented. A preferred mode of administration of Ajoene in this use is, therefore, topical.

This example also demonstrates that Ajoene can prevent infections induced by Herpesviridae (e.g., Epstein-Barr virus, cytomegaly virus, and other transforming herpesviruses). Ajoene, therefore, can protect from tumors induced by these viruses, as well as from diseases associated with herpetic infection (e.g., atherosclerosis), both in humans and animals.

Example 11

Ability of Ajoene to Prevent CD4-independent Cell-to-cell Transmission of HIV

It has been shown that adhesion mediated by identified integrins (e.g., VLA-4) is a critical step in the infection of human placental trophoblasts with HIV which is borne either by lymphocytes or by cells of lymphocytic cell lines. Experiments were, therefore, performed to ascertain whether the lymphocyte-to-trophoblast transmission of HIV can be inhibited by agents modulating the functional competence of the mediating integrins.

Human fetal placental epithelial cells, known as syncytiotrophoblasts, were organized in a monolayer and exposed to HIV-infected lymphocytic

cells and, as a negative control, to uninfected cells of the same type. This experimental system, closely simulating the actual conditions under which syncytiotrophoblasts would be exposed to HIV-infected cells *in vivo*, was used to assess integrin-modulating effects of Ajoene.

MOLT-4 clone 8 cells were labeled with calcein-AM, pretreated with different concentrations of Ajoene (0-200 micromoles per liter) and incubated with cultured term syncytiotrophoblast cells at 37°C for one hour in the presence of Ajoene. Adhesion was measured directly as fluorescence units after background correction. Controls included measuring adhesion in the presence of the appropriate carrier solvent. Without any toxic effects on cells, Ajoene inhibited adhesion of control MOLT-4 cells (not infected with HIV) to syncytiotrophoblasts with an IC_{50} of 60 micromoles per liter. At the highest concentration tested (200 micromoles per liter), the inhibition was 100%, still without toxic effects.

In order to assess the ability of Ajoene to inhibit lymphocyte-mediated HIV infection of syncytiotrophoblasts, HIV-1_{LAI}-infected MOLT-4 cells were added to adherent cultures of uninfected syncytiotrophoblasts in the presence and absence of Ajoene. Various additional control conditions were also established. First, some wells received filtered (0.45 micrometer) supernatant of cell-free HIV from infected cell cultures. Yet other negative controls included syncytiotrophoblasts cultured alone and syncytiotrophoblasts cultured with uninfected MOLT-4 cells. After 24 hours, the cocultures were washed to remove unbound lymphocytes, fixed, permeabilized, stained for viral core antigens (p24 and p55), and viewed under a fluorescence microscope.

When present in the incubation medium at a concentration of 200 micromoles per liter, Ajoene totally prevented lymphocyte-mediated infection of the syncytiotrophoblasts by HIV without toxic effects to the cells in virtually all colonies. Moreover, even in the rare instances where some fluorescence was detectable, the signal was quite weak, indicating that any infection present was negligible. Notably, in the control wells that received only cell-free HIV, infection of syncytiotrophoblast cells was also totally inhibited by Ajoene, without toxic effects, at 200 micromoles per liter and also at lower concentrations tested. Cell-free HIV infection of the syncytiotrophoblasts did occur, however, when Ajoene was absent.

This example demonstrates that Ajoene is equiactive against HIV infection transmitted by free and cell-borne viruses to CD4-negative cells, indicating that the beneficial effects of integrin modulators are not limited to a particular stage of the disease or host cell target. Furthermore, the data disclosed in this example provide further proof of the activity of Ajoene in preventing HIV infection, particularly under the conditions of sexual transmission and mother-to-fetus transmission.

Example 12

Ajoene as a Potent Anti-Shock Agent

Neutrophil aggregation within the lung microvasculature is known to be a key event in the development of adult respiratory distress syndrome (ARDS), the main cause of death in patients suffering shock. Also, administration of neutrophil-aggregating agents (such as TPA, complement anaphylotoxins or N-formyl-L-methionyl-L-leucyl-L-phenylalanine, calcium ionophore A23187, arachidonic acid, and platelet-

activating factor) to laboratory animals results in the development of ARDS. Certain inhibitors of neutrophil aggregation, such as anti-integrin monoclonal antibodies, have been shown to prevent lethality in experimental shock (see, e.g., Vedder, N.B., et al., A Monoclonal Antibody to the Adherence-Promoting Leukocyte Glycoprotein, CD18, Reduces Organ Injury and Improves Survival from Hemorrhagic Shock and Resuscitation in Rabbits, 81 *J. Clin. Invest.* 939, 1988).

The antiaggregatory activity of Ajoene disclosed in Examples 1 and 4, *supra*, is sufficiently high to indicate that Ajoene will be effective both for the prevention and treatment of various shock states (and related pathologies, such as ischemia-reperfusion injury and allograft rejection) by suppressing such aggregation.

Example 13

Ability Of Ajoene To Inhibit Fertilization

The activity of Ajoene in the hamster oocyte penetration assay was tested. This assay is routinely used to test the ability of capacitated human sperm to penetrate oocytes.

To obtain oocytes, mature hamsters (on day 1 of the estrous cycle) were injected intraperitoneally with 40 International Units (IU) pregnant mare serum gonadotropin (PMSG), followed by 40 IU human chorionic gonadotropin (HCG) 55 hours later. After 16 hours, the animals were sacrificed by intracardiac injection of 0.1 ml T61 (euthanizing agent), the oviducts were removed, and the cumulus mass was collected. Cumulus cells were removed by hyaluronidase treatment, and the freed oocytes were transferred into a watchglass containing 3 ml Biggers-Whitter-Whittingham (BWW) medium supplemented with human serum albumin (HSA)

(BWW/HSA; pH 7.5). *Zona pellucida* was removed by trypsinization for 45 seconds, after which the thus-obtained zona-free oocytes were washed three times in BWW/HSA and finally resuspended in the same medium, with appropriate additions (as described below).

Human sperm was from a donor with known fertility (No. 113). The sample (freshly taken) was allowed to liquefy, was diluted 3:1 with BWW/HSA, was centrifuged at 325 x g for 5 minutes, and was washed once with the same medium. A small portion of the original sample was analyzed (computer-assisted sperm analysis) using the Cell-Soft package. The pellet of washed sperm cells was subjected to the swim-up procedure (1 ml of the medium was added for this purpose). The swim-up was collected (about 0.5 ml), the cells were counted (Makler chamber), and their motility determined (%). The volume of the sample was adjusted with the medium to give 10^7 cells per ml, and the samples were incubated overnight (18 hours) at 37°C for capacitation. Thereafter, motility and cell density were determined once again, and the sample was divided into three equal portions, each to be subjected to appropriate treatment.

Ajoene (as a solution in ethanol) was added to separate suspensions of zona-free oocytes and capacitated sperm cells to give a final concentration of 200 micromoles per l (the final concentration of alcohol did not exceed 0.5%). Equivalent volumes of ethanol and BWW/HSA were added to the two control portions of each cell type (vehicle control and untreated cells, respectively).

After 30 minutes of separate incubation (37°C) as per protocol above, sperm cells and oocytes subjected to the same treatment were brought into contact in 6-well culture plates, and the plates were transferred to an air incubator for an additional 3

hours (37°C). Insemination was stopped by transferring oocytes from each well onto watchglasses, where the eggs were allowed to settle and were freed from excess sperm. Thereafter, slide-coverslip preparations were prepared from the contents of each watchglass and viewed under a phase-contrast microscope (x 40). Penetration was calculated by relating the number of penetrated oocytes to the total number of oocytes (values expressed as percentages).

Both intact and vehicle-pretreated gametes showed a high level of penetration (96.2% and 100%, respectively). Conversely, pretreatment with Ajoene inhibited penetration completely (0%).

It should be emphasized that the effect of Ajoene was not associated with toxicity or impaired cellular vitality (judging by intact motility of ajoene-pretreated sperm cells). All results were reliably reproduced (coefficient of variation did not exceed 15%).

In vivo, in humans and other mammals, sperm cannot penetrate or fertilize oocytes without first becoming capacitated. Capacitation ordinarily occurs in the genital tract of the human female. Once the species-specific *zona pellucida* has been removed from a hamster oocyte, however, the ability of capacitated human sperm to penetrate the zona-free hamster oocyte becomes a precise simulation of the *in vivo* ability of the same capacitated sperm to penetrate a human oocyte, as in the case of fertilization. It is, therefore, highly likely that Ajoene would exhibit the same activity in *in vivo* use as seen in this *in vitro* assay.

This example underscores once again the ability of Ajoene to modulate the adhesion and fusion function of integrins, and it also provides further proof that diseases and conditions associated with

undesirable fusion can be prevented and treated with integrin modulators.

Example 14

5 Ajoene as an Antimetastatic Agent

Experimental metastasis

A suspension of 10^5 melanoma B16 cells was pretreated for 30 min with Ajoene, and the suspension (including Ajoene) was injected intravenously into
10 male C57BL/6 mice. The mice were sacrificed 3 weeks post-injection, and their lungs subjected to microscopic examination. Ajoene prevented lung colonization at 200 micromoles per liter.

Melanoma cell implantation

15 Male C57BL/6 mice were inoculated subcutaneously with 10^6 B16 cells (pretreated for 15 min with Ajoene) in their pretreatment medium, and the volume of the tumors formed was measured 14 and 21 days after the injection. Implantation was inhibited with an IC_{50} of
20 2.5 micromoles per liter.

Example 15

Ajoene and Disintegrins Suppress Fusion Leading to Syncytium Formation in HTLV-I-infected Cells

25 MT-2 cells harbor the complete genome of human T cell lymphotropic virus type 1 (HTLV-I), and they transmit the virus to susceptible uninfected cells in cocultures. The transmission manifests itself as fusion of MT-2 cells with the uninfected
30 cells, followed by syncytium formation. K562 cells transfected with VCAM-1, a ligand of the integrin VLA-4, were used as a fusion partner for MT-2 cells. MT-2 and K562/VCAM-1 cells were washed and resuspended in complete RPMI medium at a density of 2×10^6 /ml. MT2
35 cells were seeded into flat-bottom 96-well microtiter plates (100 microliters per well). Serial dilutions

of Ajoene or of the disintegrin kistrin (final concentration) were introduced into the wells, and the plate was incubated for 30 min prior to addition of 100 microliters of K562/VCAM-1 cells. The plates were then incubated for 4 h before scoring syncytium formation.

Ajoene inhibited syncytium formation with an IC_{50} of ~ 0.6 micromoles per liter and an IC_{100} of 4.3 micromoles per liter. Kistrin inhibited syncytium formation by $\sim 40\%$ at 3.4 micromoles per liter; higher concentrations were not tested.

This example provides conclusive proof that the ability of Ajoene to prevent virus transmission cell-to-cell is not limited to HIV infection. Therefore, infections that are caused by other cell-borne viruses will likewise be inhibited by Ajoene *in vivo*. Thus, Ajoene can be used to prevent or treat a wide variety of viral infections in humans and animals.

Furthermore, when viewed in conjunction with the data of Examples 8 and 13, the result obtained herein indicates that Ajoene and like substances can prevent or attenuate adhesion and fusion events associated with a wide variety of conditions, diseases, and illnesses, whether induced by viruses, associated with inflammatory processes, or physiological.

Of equal importance, this example demonstrates that kistrin, a disintegrin modulating $\beta 1$, $\beta 2$, and $\beta 3$ integrins, inhibits HTLV-I-mediated cell fusion.

Example 16In Vivo Modulation by Disintegrins of LFA-1-
and VLA-4-mediated Recruitment of Leukocytes
in Murine Models of Human Autoimmune Diseases

5 Nonobese diabetic (NOD) mice are
characterized by spontaneous development of insulinitis
(similar to human insulin-dependent diabetes) and
sialoadenitis (similar to Sjögren's disease). The
development of autoimmune inflammation in these
10 animals involves several integrin receptors, including
VLA-4, CR3, and LFA-1 (CD11a/CD18). Lymphocyte
infiltration into the pancreata is mediated primarily
by VLA-4, whereas the predominant mechanism of
lymphocyte recruitment into the salivary glands
15 involves LFA-1.

NOD mice (age, 3 to 4 weeks old; average
body weight, 10 g) were divided into three groups.
Experimental animals received intraperitoneal
injections of disintegrins (0.3 mg/kg per animal every
20 other day) for 9 weeks. Control mice were
administered with the vehicle.

The mice were sacrificed and subjected to
autopsy. The pancreata and salivary glands were
removed and frozen (-70°C). Thin sections (5
25 micrometer thick) of these organs were fixed in
acetone, stained with hematoxylin and eosin, and
evaluated microscopically for lymphocyte infiltration
(Tables 6 and 7).

Table 6. Effects of disintegrins on the spontaneous development of autoimmune diabetes

Treatment	Number of mice with infiltration scores:			
	-	+	++	+++
Control	0	1 (12.5%)	3 (37.5%)	4 (50%)
Eristostatin	1 (20%)	4 (80%)	0	0
Kistrin	3 (60%)	2 (40%)	0	0

Infiltration score: (-) no infiltrating lymphocytes; (+) 1-3 infiltrated islets; (++) 4 islets with moderate infiltration and/or 1-2 islets with large infiltrates; (+++) 3 or more large infiltrates.

Table 7. Effects of disintegrins on the spontaneous development of autoimmune sialoadenitis

Treatment	Number of mice with infiltration scores:			
	-	+	++	+++
Control	1 (14.3%)	2 (28.6%)	3 (42.9%)	1 (14.3%)
Eristostatin	0	2 (40.0%)	3 (60.0%)	0
Kistrin	2 (40.0%)	2 (40.0%)	0	1 (20.0%)

Infiltration score: (-) no infiltrating lymphocytes; (+) 1-3 small foci involving only one duct; (++) small foci and/or 1 large focus involving several ducts; (+++) 2 or more foci involving several ducts.

Eristostatin and kistrin increased the proportion of animals with mild infiltration of pancreatic islets and completely prevented the development of large infiltrates (lack of high scores; see, Table 6). Kistrin tended to be more active in this respect, judging from the higher percentage of animals with complete absence of infiltration. Similar effect was observed in the case of sialoadenitis (Table 7).

This example demonstrates that the recruitment of leukocytes to the tissues and the resulting development of autoimmune inflammation is

dramatically retarded by modulation of $\beta 1$ and $\beta 2$ integrins with disintegrins. Thus, a broad range of autoimmune diseases (e.g., diabetes, Sjögren's syndrome, lupus, and rheumatoid arthritis) can be
5 treated by modulators of $\beta 1$ and $\beta 2$ integrins.

Moreover, other types of destructive inflammation known to arise from excessive integrin-mediated recruitment of leukocytes (e.g., shock, ischemia-reperfusion injury, and graft rejection) will
10 likewise be amenable to treatment with disintegrins.

Data disclosed in this example and Example 15, *supra*, indicate that disintegrins offer an approach to treatment of viral diseases, particularly in cases where the transmission of the virus occurs
15 predominantly cell-to-cell and/or involves cell fusion. Other conditions, diseases, and illnesses, associated with excessive or otherwise undesirable cell fusion can, therefore, be treated by disintegrins with beneficial effects. Thus, disintegrins can be
20 used as pharmaceuticals in various contexts, including, without limitation, the fields of infectious diseases, autoimmune diseases, shock and resuscitation from shock, transplantation (prevention of allograft rejection and GVHD), and reproductive
25 biology.

Taken together, the examples clearly indicate that Ajoene modulates several integrins belonging to $\beta 1$, $\beta 2$, $\beta 3$, and $\alpha 4$ families of integrins.
30 Notably, the modulating effects measured *in vitro*, such as inhibition of neutrophil aggregation (Example 1) and lymphocyte adhesion (Examples 4 and 5), are reproduced under *in vivo* conditions. As Examples 6 and 7 demonstrate, several inflammatory responses are
35 significantly attenuated as a result of *in vivo* integrin modulation by Ajoene. Similarly, inhibition

of VLA-4-dependent melanoma cell adhesion, observed *in vitro* (Example 3), takes place *in vivo* as well, judging by the suppression of melanoma metastasis (Example 14). The ability of Ajoene to modulate *in vivo* $\beta 1$, $\beta 2$, $\beta 3$, and $\alpha 4$ integrins strongly indicates that it can be used as a therapeutic agent in various contexts. Thus, Ajoene can be used in a range of clinical situations; it can be used to treat diseases, the pathogenesis of which is strongly integrin-dependent (e.g., HIV infection, shock, and inflammatory/autoimmune pathologies) and diseases and illnesses in which only certain stages or manifestations exhibit a dependence on integrin function. In addition, Ajoene can be used in the treatment of diseases with unknown etiology if these diseases involve an integrin-mediated aspect, such as inflammation. In this latter use Ajoene will mitigate the symptoms of the disease and alleviate the condition of the patients.

Furthermore, as demonstrated in Example 16 above, *in vivo* modulation of $\beta 1$, $\beta 2$, $\beta 3$, and $\alpha 4$ integrins by disintegrins produces the same therapeutic benefits as those described above for Ajoene. Thus, the same diseases, conditions and illnesses that Ajoene can treat are also treatable by disintegrins. In general, any non-antibody substance herein defined as a modulator of several integrin receptors (e.g., of a $\beta 2$ integrin, such as LFA-1, and of a $\beta 1$ or $\alpha 4$ integrin, such as VLA-4) will exhibit the same range of activities *in vitro* and *in vivo* as Ajoene and certain disintegrins (e.g., kistrin or eristostatin).

In the context of the pronounced homologies among various members of the integrin superfamily, the ability of Ajoene and disintegrins to act on integrins of distinct subfamilies clearly indicates that other

integrins, not mentioned in the above Examples, can likewise be modulated with Ajoene and disintegrins to produce yet further therapeutic benefits. Therefore, the scope of this invention goes beyond specific

5 pathologies involving $\beta 1$, $\beta 2$, $\beta 3$, and $\alpha 4$ integrins.

In order to achieve maximum benefit in treating a particular disease with modulators like Ajoene and disintegrins (which modulators act on several integrin receptors), it may be reasonable to

10 use various targeted modes of administration. Methods of targeting pharmaceutical agents (and other substances) to desired locations, such as specific cells or organs, are well known in the art and described in numerous publications. One such method

15 involves loading an integrin modulator into liposomes that contain on their surface antibodies against certain tissue markers, although other targeting techniques (including other targeting techniques based on antibodies) can also be used.

20 The above description has been offered for illustrative purposes only and is not intended to limit the scope of the invention of this application, which is as described in the claims below.

WE CLAIM:

1. A method of treating a viral infection comprising administering an effective amount of a disintegrin, or a variant or analog thereof, to an animal suffering from such an infection.
2. The method of Claim 1 wherein the viral infection is caused by a retrovirus.
3. The method of Claim 2 wherein the viral infection is caused by HIV.
4. The method of Claim 2 wherein the viral infection is caused by HTLV-1.
5. The method of Claim 1 wherein the viral infection is caused by a virus of the Herpesviridae family.
6. The method of Claim 5 wherein the viral infection is caused by herpes simplex.
7. The method of Claim 1 wherein the viral infection is hepatitis.
8. The method of Claim 1 wherein the disintegrin is administered intralymphatically.
9. The method of Claim 1 wherein the disintegrin is administered topically.
10. The method of Claim 9 wherein the disintegrin is administered intravaginally.

11. A method of treating a viral infection comprising administering to an animal suffering from such an infection a disintegrin, or a variant or analog thereof, in an amount effective to inhibit
5 replication of the virus.

12. The method of Claim 11 wherein the viral infection is caused by a retrovirus.

10 13. The method of Claim 12 wherein the viral infection is caused by HIV.

14. The method of Claim 12 wherein the viral infection is caused by HTLV-1.

15 15. The method of Claim 11 wherein the viral infection is caused by a virus of the Herpesviridae family.

20 16. The method of Claim 15 wherein the viral infection is caused by herpes simplex.

17. The method of Claim 11 wherein the viral infection is hepatitis.

25 18. A method of treating an animal comprising administering to the animal an amount of a disintegrin, or a variant or analog thereof, which is effective to inhibit transfer of genetic material to a
30 cell in the animal.

19. The method of Claim 18 wherein the genetic material is transferred from one cell to another cell.

35

20. The method of Claim 18 wherein the genetic material is viral genetic material.

21. The method of Claim 20 wherein the viral genetic material is that of a retrovirus.

22. The method of Claim 21 wherein the viral genetic material is that of HIV.

23. The method of Claim 21 wherein the viral genetic material is that of HTLV-1.

24. The method of Claim 20 wherein the viral genetic material is that of a virus of the Herpesviridae family.

25. The method of Claim 24 wherein the viral genetic material is that of herpes simplex virus.

26. The method of Claim 20 wherein the viral genetic material is that of a hepatitis virus.

27. The method of Claim 19 wherein the transfer of genetic material occurs at least in part by means of syncytia formation.

28. A method of preventing a viral infection of uninfected cells comprising contacting the uninfected cells with an agent which is an integrin modulator in an amount which is effective to inhibit adhesion of the virus to the cells.

29. The method of Claim 28 wherein the contacting takes place in an animal, the agent being administered to the animal in an amount which is

effective to inhibit adhesion of the virus to the cells.

30. The method of Claim 28 or 29 wherein
5 the virus is a retrovirus.

31. The method of Claim 30 wherein the
retrovirus is HIV.

10 32. The method of Claim 30 wherein the
retrovirus is HTLV-1.

33. The method of Claim 28 or 29 wherein
the virus is a virus of the Herpesviridae family.
15

34. The method of Claim 33 wherein the
virus is herpes simplex virus.

35. The method of Claim 28 or 29 wherein
20 the virus is a hepatitis virus.

36. The method of any one of Claims 28-35
wherein the agent is a disintegrin or a variant or
analog thereof.
25

37. The method of any one of Claims 28-35
wherein the agent is ajoene.

38. A method of treating shock comprising
30 administering to an animal suffering from shock an
effective amount of an agent which is an integrin
modulator.

39. The method of Claim 38 wherein the
35 agent is a disintegrin, or a variant or analog
thereof.

40. A method of treating inflammation comprising administering to an animal suffering from inflammation an effective amount of an agent which is an integrin modulator.

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41. The method of Claim 40 wherein the inflammation is chronic.

42. The method of Claim 41 wherein the
10 chronic inflammation is caused by a chronic infection.

43. The method of Claim 42 wherein the chronic infection is tuberculosis.

15 44. The method of Claim 43 wherein the agent is administered by inhalation.

45. The method of Claim 41 wherein the
20 chronic inflammation is associated with sarcoidosis.

46. The method of Claim 40 wherein the inflammation is acute.

47. The method of Claim 40 wherein the
25 inflammation is phlebitis, peritonitis or meningitis.

48. The method of Claim 40 wherein the
inflammation is associated with ischemia reperfusion
injury.

30

49. The method of Claim 40 wherein the inflammation is associated with an immune response.

50. The method of any one of Claims 40-49
35 wherein the agent is a disintegrin, or a variant or analog thereof.

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51. The method of any one of Claims 41-49 wherein the agent is ajoene.

52. A method of treating arthritis
5 comprising administering to an animal suffering from arthritis an effective amount of an agent which is an integrin modulator.

53. The method of Claim 52 wherein the
10 arthritis is rheumatoid arthritis.

54. The method of Claim 52 or 53 wherein the agent is ajoene.

55. The method of Claim 52 or 53 wherein
15 the agent is a disintegrin, or a variant or analog thereof.

56. The method of Claim 52 wherein the
20 agent is administered topically.

57. A method of contraception in a mammal comprising administering to the mammal an effective amount of an agent which modulates integrins.

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58. The method of Claim 57 wherein contraception occurs as a result of inhibiting fertilization of an egg by sperm by contacting the egg, the sperm, or both with the agent.

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59. The method of Claim 58 wherein contraception occurs as a result of inhibiting chemotaxis of sperm.

60. The method of Claim 58 wherein
35 contraception occurs as a result of inhibiting sperm

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interactions with the epithelium of the female genital tract.

61. The method of Claim 57 wherein
5 contraception occurs as a result of preventing
implantation of an embryo.

62. The method of Claim 57 wherein
contraception occurs as a result of inhibiting sperm
10 or sperm development and maturation in a male animal.

63. The method of any one of Claims 57-62
wherein the agent is ajoene.

15 64. The method of any one of Claims 57-62
wherein the agent is a disintegrin, or a variant or
analog thereof.

65. The method of Claim 57 wherein the
20 agent is administered vaginally.

66. The method of Claim 57 wherein the
agent is administered orally.

25 67. The method of Claim 57 wherein the
agent is administered topically to a mucous membrane.

68. A method of suppressing an immune
response in a mammal comprising administering an
30 effective amount of an agent that modulates integrins
to the mammal.

69. The method of Claim 68 wherein the
immune response is an allergic reaction.

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70. The method of Claim 68 wherein the immune response is allograft rejection.

5 71. The method of Claim 68 wherein the immune response is graft versus host disease.

72. The method of any one of Claims 68-71 wherein the agent is ajoene.

10 73. The method of any one of Claims 68-71 wherein the agent is a disintegrin, or a variant or analog thereof.

15 74. A method of treating an autoimmune disease in a mammal comprising administering an effective amount of an agent that modulates integrins to the mammal.

20 75. The method of Claim 74 wherein the autoimmune disease is rheumatoid arthritis, insulin-dependent diabetes mellitus, or Sjogren's syndrome.

25 76. The method of Claim 74 or 75 wherein the agent is ajoene.

77. The method of Claim 74 or 75 wherein the agent is a disintegrin, or a variant or analog thereof.

30 78. A method of treating an animal comprising administering to the animal an effective amount of a disintegrin, or variant or analog thereof, which inhibits undesirable integrin-mediated cell-cell fusion.

79. The method of Claim 78 wherein the cell-cell fusion results in giant multinucleated cell formation and the disintegrin, or variant or analog thereof, inhibits the giant multinucleated cell formation.

80. The method of Claim 79 wherein the cell-cell fusion results in syncytia formation and the disintegrin, or variant or analog thereof, inhibits the syncytia formation.

81. The method of Claim 78 wherein the cell-cell fusion results in osteoclast formation and the disintegrin, or variant or analog thereof, inhibits the osteoclast formation.

82. The method of Claim 78 wherein the cell-cell fusion is associated with granuloma formation and the disintegrin, or variant or analog thereof, inhibits the granuloma formation.

83. A method of treating an animal comprising administering to the animal an amount of ajoene effective to inhibit undesirable integrin-mediated cell-cell fusion which results in osteoclast formation.

84. A method of treating an animal comprising administering to the animal an amount of ajoene effective to inhibit undesirable integrin-mediated cell-cell fusion which is involved in granuloma formation.

85. A method of inhibiting the formation of lesions in an animal comprising administering to the

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animal an effective amount of an agent which is an integrin modulator.

5 86. The method of Claim 85 wherein the agent is a disintegrin, or a variant or analog thereof.

10 87. The method of Claim 85 wherein the agent is ajoene.

15 88. A method of treating psoriasis comprising administering to an animal suffering from psoriasis an effective amount of an agent which is an integrin modulator.

 89. The method of Claim 88 wherein the agent is a disintegrin, or a variant or analog thereof.

20 90. The method of Claim 88 wherein the agent is ajoene.

25 91. A method of treating atherosclerosis comprising administering to an animal suffering from atherosclerosis an effective amount of an agent which is an integrin modulator.

30 92. The method of Claim 91 wherein the agent is a disintegrin, or a variant or analog thereof.

 93. The method of Claim 91 wherein the agent is ajoene.

35 94. A method of treating a disease or a condition involving a plurality of integrin-dependent

etiopathogenetic mechanisms comprising inhibiting a plurality of the mechanisms by administering to an animal suffering from such a disease a safe and effective amount of an integrin modulating agent.

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95. The method of Claim 94 wherein the mechanisms include a viral infection.

96. The method of Claim 95 wherein the mechanisms further include an autoimmune reaction.

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97. The method of Claim 95 wherein the mechanisms further include inflammation.

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98. The method of Claim 94 wherein the disease is atherosclerosis.

99. The method of any one of Claims 94-98 wherein the agent is a disintegrin, or a variant or analog thereof.

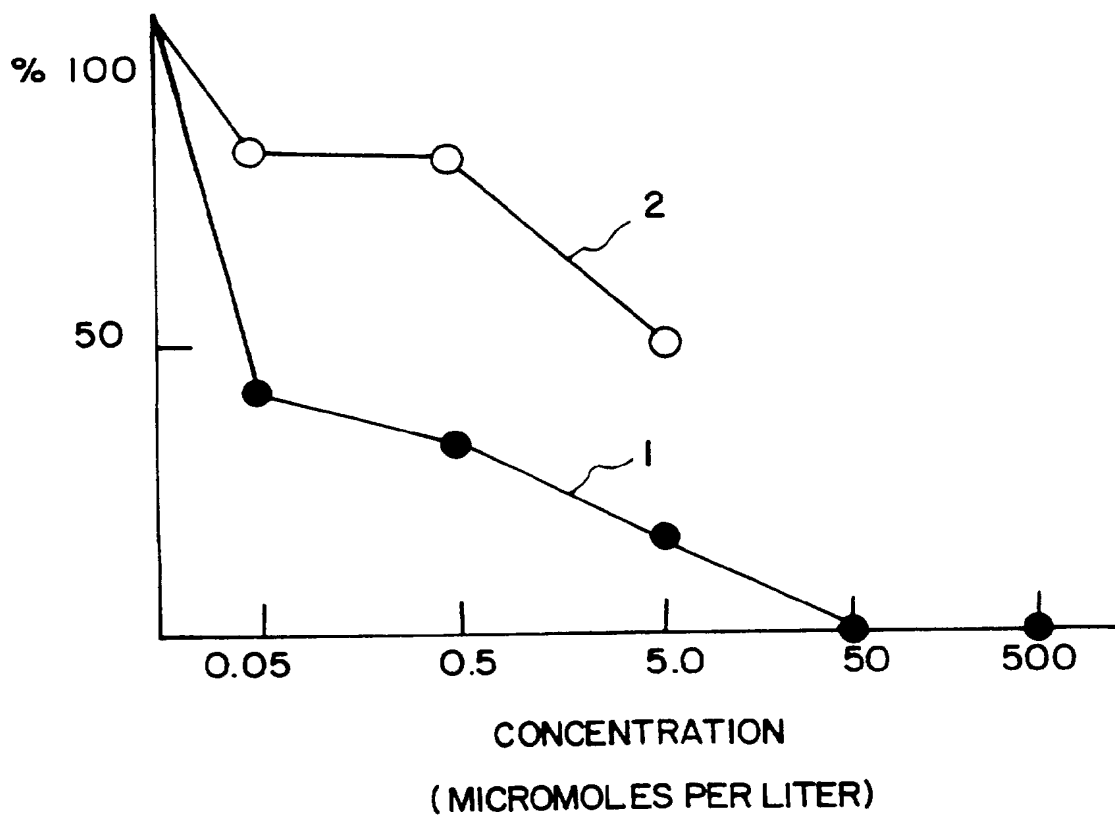
20

100. The method of any one of Claims 94-98 wherein the agent is ajoene.

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FIG.1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00583

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/10
US CL : 514/708

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/708

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN; Compounds and methods of use

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Vol. 104, 1986, TSAI et al, "Antiviral properties of garlic: in-vitro effects on influenza B, herpes simplex and coxsackie viruses", page 24, abstract no. 104:61572a, see entire abstract.	1-37, 78-83, 94-95, 99-100
Y	Planta Medica, Vol. 57, 1991, LAWSON et al, "Identification and HPLC Quantification of the Sulfates and Dialk(en)yl Thiosulfinates in Commercial Garlic Products", Pages 363-370, see pages 363-370.	1-37, 78-83, 94-95, 99-100
Y	Planta Medica, Vol. 58, 1992, WEBER et al, "In Vitro Viricidal Effects of Allium sativum (Garlic) Extract and Compounds", pages 417-23, see pages 417-423.	1-37, 78-83, 94-95, 99-100

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

15 MAY 1997

Date of mailing of the international search report

06 JUN 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00583

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Vol. 111, 1989, NAKAGAWA et al, "Prevention of liver damage by aged garlic extract and its components in mice" page 61, abstract no. 111:146760e, see entire abstract.	5, 17, 26, 35
Y	Proceedings National Academy of Science, USA, Vol. 88, July 1991, DOUVAS et al, "Multiple overlapping homologies between two rheumatoid antigens and immunosuppressive viruses", pages 6328-6332, see pages 6328-6332.	2-4, 12-14, 21-23, 30-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00583

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-37, 78-83, 94-95 and 99-100

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.